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AWARD NUMBER DAMD17-97-1-7245

TITLE: Butyrate Therapy for Poorly Differentiated Breast Cancer

PRINCIPAL INVESTIGATOR: John McBain, Ph.D.

CONTRACTING ORGANIZATION: Dartmouth College
Hanover, New Hampshire 03755-3580

REPORT DATE: October 1998

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 1998	Annual (15 Sep 97 - 14 Sep 98)

4. TITLE AND SUBTITLE

Butyrate Therapy for Poorly Differentiated Breast Cancer

5. FUNDING NUMBERS

DAMD17-97-1-7245

6. AUTHOR(S)

John McBain, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Dartmouth College
Hanover, New Hampshire 03755-3580

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research And Materiel Command
ATTN: MCMR-RMI-S
504 Scott Street
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

This research program is directed at providing a 24 hr regimen of butyric acidemia which exceeds 5 mM butyrate while remaining below 15 mM. Work to date has shown that appropriate dose schedules of treatment with methylenecyclopropane acetic acid can inhibit butyrate catabolism sufficient for this end. The inhibition effected by MCPA has been shown to be both transitory and suppressed by high conentrations of butyrate, suggestive of some challenging pharmacological interactions between MCPA and butyrate. Butyrate (free from exogenous counterion) is shown to be available from tributyrin, although the water insolubility of tributyrin limits its usefulness. Mice have been found to tolerate butyrate concentrations as high as 58 mM and accompanying pH's as low as 6.9. The metabolic acidosis and hypoglycemic sequelae require incremental correction with bicarbonate and glucose, while MCPA-induced hypothermia requires temperature control of housing. The goal of these studies is the demonstration of histone hyperacetylation in the tissues of these mice, and then whether human breast cancer cell line xenografts can be selectively caused to regress. It is anticipated that our goals will be met by a combination of continuous infusion of monobutyrin (from which butyrate is freely available) to animals which are initially pretreated with MCPA, and then provided MCPA as a continuous supplement.

14. SUBJECT TERMS			15. NUMBER OF PAGES
Breast Cancer	butyrate	histone hyperacetylation	10
acidosis	therapy	pharmacokinetics	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

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John R. Bair 13 Oct. 98

PI - Signature Date

DoD IDEA Grant - Butyrate therapy for poorly differentiated breast cancer

Congressionally mandated breast cancer research program

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Introduction

The research proposed was directed at the prediction that treatment of mice with MCPA (methylenecyclopropane acetic acid), an inhibitor of the SCAD (short-chain acyl CoA dehydrogenase) enzyme of butyrate metabolism, would allow reasonable doses of butyrate ester to be used to provide millimolar concentrations of butyrate to persist in circulation for the 24 hr predicted to be necessary to effect regression of butyrate-sensitive breast cancers (such as the SKBr-3 cell line). The work to date has upheld this prediction, but has also pointed out shortcomings of the widely touted butyrate prodrug, tributyrin, and of the assumption that a single dose of MCPA (which brings about a protracted isovaleric acidemia in malnourished children) would suffice to provide 24 hr of relative freedom from rapid catabolism of butyrate. The findings have however suggested several prospective solutions to these problems.

Previous attempts at instituting butyric acidemia have made use of either salts of esters of butyrate administered by a variety of routes. Most recently, tributyrin oil has been given orally to human volunteers in phase 1 trials seeking estimates of maximally tolerated dose (1). In all studies to date, the limiting factor has been the very rapid consumption of free butyrate (typically less than 10 minutes to clear butyrate from blood after an I.V. bolus dose). Thus, in all published papers to date, peak levels of butyrate have rarely exceeded 1 mM, and this peak level is rarely maintained for 10 minutes. The exception has been tributyrin, which as an oil-in-water emulsion administered intraperitoneally provided millimolar concentrations in a pilot trial (Egorin, unpublished). The rapid consumption of released butyrate, which is the ultimate inhibitor of histone deacetylase, would still presumably have limited its effectiveness. Thus, full reports have to date been limited to the effects of sub-millimolar plateau concentrations of butyrate.

Body

Tolerance of agents - We initially set out to determine the tolerance of mice for MCPA and tributyrin. MCPA had formerly been studied as a toxin, having been considered as the agent responsible for Jamaican vomiting sickness, an illness which causes either acute distress and death or hepatic necrosis and other sequelae (2). Guided by previous work, we had available both cage heaters and cloth/wood chip accouterments; indeed, the first dose we chose caused a precipitous fall in rectal temperature which was reversed by these accommodations. While both MCPA and tributyrin emulsion caused only mild changes in activity, the combination of a 30 mg/kg dose of MCPA and a 2 g/kg dose of tributyrin (as a 20% emulsion stabilized with 10% glycerol and 0.01% of both Pluronic F68 and Tween 80) caused loss of consciousness and pronounced Kussmaul breathing. These mice typically recover consciousness after about 2 hr, and thereafter begin feeding and are indistinguishable from non-treated mice. The effects attributed to acidosis and hypoglycemia are markedly ameliorated using subcutaneous sodium bicarbonate and glucose.

Method of butyrate determination - The methods used to determine butyrate concentrations in the plasma of these mice use a published method (3), which basically exploits the denaturation and release of acids from serum proteins, direct extraction into ethyl ether, back extraction into dilute alkali, and injection of acidified sample onto a methylsilicone silica-packed column run with hydrogen carrier gas, and detected by flame ionization. Detection limits are less than 100 μM, linearity of quantitation is excellent, and recovery from serum or plasma is in excess of 95%over the range 500 μM to 50 mM. We employ an internal standard (2-ethylbutyric acid), mainly to verify

recovery. Tributyrin is itself retained in the ether phase, but upon adding emulsified tributyrin to human fresh serum, butyrate is rapidly produced, likely as a result of the action of endogenous esterases.

Effect of agents on circulating butyrate - We have determined the optimum doses for these agents, and have settled upon 25 mg MCPA / kg and between 0.4 and 2 g / kg tributyrin. Plasma butyrate concentrations, after intraperitoneal administration of 20-50% of the predicted lethal dose of tributyrin (4 g / kg), peak at 2-20 mM within 30 minutes of administration. As shown in fig. 1, MCPA pretreatment results in an increase of this peak concentration by about 3 fold. However the major effect of MCPA is in allowing maintenance of plasma butyrate concentrations at levels in excess of 5 mM for 2 hours. During this time, the MCPA pretreated mice remained unconscious, while the mice given tributyrin alone regained consciousness within one hour of administration.

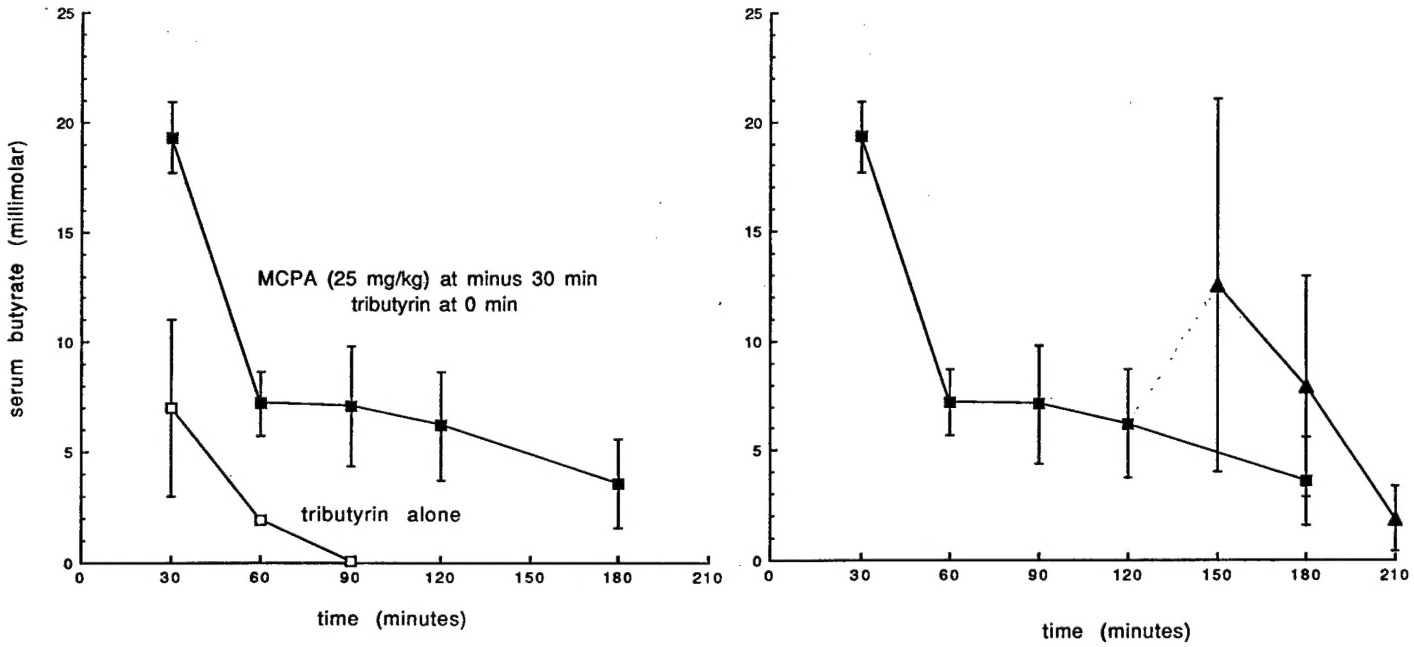

Figure 1. Effect of MCPA pretreatment on serum butyrate concentrations following a single or repeated administration of tributyrin emulsion. Some mice were injected intraperitoneally with MCPA (25 mg/kg) at 30 minutes prior to administration of tributyrin emulsion (2 g/kg) at the start of the experiment (closed squares). As represented by the open squares, other mice received tributyrin alone. After 120 minutes, some animals received additional tributyrin (closed triangles; same dose). At the times indicated, mice were anaesthetized and terminally bled from the axillary artery. Serum was extracted and the organic acid fraction examined by gas chromatography. Materials eluting after a time characteristic of butyric acid were quantitated in comparison with a dilution series of butyric acid. Data are average and range for two mice each.

Transitory effectiveness of single administration of MCPA - The schedule of administration of these agents markedly determine effectiveness. MCPA-treated mice regained the ability to catabolize butyrate, evident as early as 2 hr after administration

and nearly completely by 9 hours. In addition, a preexisting butyric acidemia decreased the effectiveness (both initial and ultimate) of MCPA given concurrently. A 30 minute pretreatment is used routinely, although this has not been determined to be optimum. The overriding hypothesis is that a given dose of MCPA varies in effectiveness depending upon competing butyrate concentrations. MCPA CoA is mechanistically a suicide inhibitor of SCAD (4), but butyrate and MCPA (or their CoA esters) are also likely to be competitive inhibitors of binding to CoA transferases, transmembrane transport proteins and SCAD itself.

Inter-experiment and intra-experiment variability in pharmacokinetics - A particularly troubling finding, which we propose to be related to tributyrin pharmaceutics, is the sometimes extreme variation among replicate samples taken from mice given tributyrin emulsion. It has not been uncommon to find 5-fold variation between butyrate levels in mice treated apparently identically with a given dose of tributyrin emulsion. In these samples, both the internal standard and endogenous 5.5 min peak (presumptive isovaleric acid) are typically within 10% of each other. In addition, the level of Kussmaul breathing and the level of consciousness (which correlates well with serum butyrate) can vary within groups. At least 3 sources of variability can be inferred:

1. Method of emulsion preparation - Freshly prepared emulsion has been found to confer lethal effects which are not seen in those which are allowed to 'age' overnight in the refrigerator. Although the presumptive oil microdroplets settle within hours of standing, the emulsion stops sort of coalescing into larger droplets, and remains as a colloidal suspension, although apparently less than 10% water. Better reproducibility is obtained, but concern remains.
2. Syringe filling technique - Intraexperimental variability can be partially ascribed to the tendency of the suspension to segregate within a filled syringe; better results are obtained if the syringe is filled immediately before use (and not 20 seconds after filling, or after 2 mice have already been injected with the same syringe).
3. Residual variation in bioavailability - There remains an element of variation which may relate to the combination of tributyrin insolubility and the peritoneal environment. This is readily observable by comparing over time the respiratory activity of animals which had the same age and weight and treatment, but had differing levels of response.

We conclude that tributyrin is a problematic agent for parenteral administration; preliminary trials of cyclodextrin-stabilized tributyrin indicated little improvement. (see Modifications to research plan, below).

Remediation of acidosis - Besides cage warming, correction of blood pH has proven essential, especially when the trial exceeds 2 hours and includes a repeat administration of MCPA and tributyrin. Within 30 minutes of tributyrin administration to MCPA-pretreated mice, a coma-like state of unconsciousness and Kussmaul breathing (rapid, deep inspiration) is seen, which persists for 1-3 hr in the absence of remediation. As mentioned, this effect is seen when butyrate concentrations exceed 5 mM. While blood pH falls as low as 6.88, typical pH values are nearer 7.1 in the unconscious MCPA/tributyrin-treated mice. Correction of the acidosis with sodium bicarbonate corrects the acidosis and substantially calmed the Kussmaul breathing without significantly altering the apparent level of sensorium or the serum butyrate concentration. Glucose is included with sodium bicarbonate for this subcutaneous fluid therapy, resulting in partial correction of both hypoglycemia and protein catabolism (evident from the level of presumptive isovaleric acid).

Modifications to original research plan

Our plans to test butyrate therapy require control (or at least thorough knowledge) of butyrate concentrations in the mice. The measure of histone acetylation, which will mark the point when we can address the effectiveness of the therapy against normal and cancer cells, is essentially a running summation of the lower limit of butyrate concentrations in the given cells, a summary which ignores (or is indifferent to) the problematic rises of butyrate to high levels. As before, our goal remains the maintenance of not less than 3-5 mM butyrate for a continuous 24 hr period, but is now supplemented to exclude rises to over 15 mM. The experience with tributyrin is such that hourly injections of close to an LD50 would be required (a frequency which is itself frowned upon by members of animal use oversight committees) and this would be accompanied by over 20 such paroxysmal exacerbations of the acidemia/acidosis.

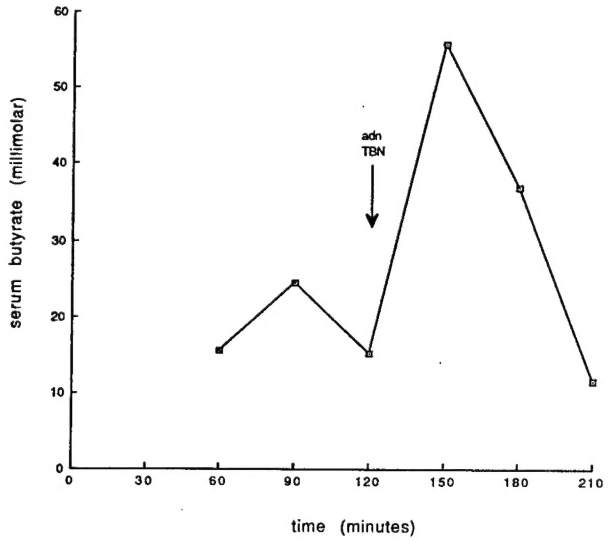

Figure 2. Extreme butyric acidemia following repeat injection of tributyrin in MCPA-pretreated mice. Experimental protocol was identical to the experiment depicted in figure 1. Single mouse/point.

Such transitory but severe increases in acidemia would likely limit the tolerability of the therapy, possibly inducing Reye syndrome-like changes in liver and nervous system. In order to derive a rational assessment of the therapeutic index, we will need better control of butyrate concentrations and general acidosis (as an electrolyte and blood gas derangement). To this end we are proposing to:

Shift to a water-soluble butyrate ester. Of two possibilities, we favor monobutyrin (glycerol monobutyrate) which has been shown to be well tolerated as well as providing caloric nutrition for rats (27 g/kg/day for 7 days, ref. 5) and dogs. It has been given intravenously. An alternative proprietary formulation, monoacetone glucose 3-butyrate, has been extensively tested by the French group which synthesized and patented it (6). I have longstanding approval from their representatives if I would wish to use the agent, or a congener. In either case, the ester would be administered by continuous infusion, using a Harvard Instruments syringe pump which has been modified to accommodate 12 syringes. We have provisional approval of the Institutional Animal Care and Use Committees of both Dartmouth College and the VA Research Service for use

of a peritoneal catheter (held in place with an acrylate adhesive) and infusion pump. The therapy will be designed to continuously maintain unconsciousness (as well as relief of acidosis).

Intermittently determine blood pH, pCO2 and (if possible) total volatile organic acids. We have designed and begun construction of an analyzer for determination of acid/base parameters on 50 µl of blood, allowing sampling of mice without killing them. Thus far we have had to rely on pH determinations alone, calculating bicarbonate from certain assumptions. In addition, a butyrate concentration of 20-50 mM would theoretically contribute an error to CO2 determinations. If the device works as well as predicted, we should be able to rapidly assess dose modifications and provide better care for the animals.

Continuously monitor respiratory activity and consciousness, to be used eventually as an index of the need to increase administration of bicarbonate or butyrate ester, respectively. The VA Research Service has recently obtained a set of MacLab A/D converters for computer monitoring and recording of instrument outputs. Using an existing bioamplifier and transducers, we will monitor respiratory activity and leg movement of animals held in harnesses within temperature-controlled housing. Infusion rates and proportions will be modified (and recorded on-line) based upon chemical analyzer results and the physiological responses of representative animals.

Conclusions

The feasibility of butyrate therapy using a combination of a butyrate glyceride and an inhibitor of its catabolism (e.g., MCPA) has been provisionally upheld. While previous investigators published work demonstrating high micromolar butyrate concentrations with time courses of minutes or dependence on continuous consumption or infusion, we are able to maintain concentrations in excess of 5 mM for hours. Furthermore, our regimen could provide peak butyrate concentrations in the 20-60 mM range, with blood pH <7, and the mice still recovered fully. Remaining problems which require solutions are the poor pharmaceutical properties of tributyrin (a water-insoluble oil), including high variability which is compounded by repetitive dosing with the butyrate ester. Additional problems include the need to administer MCPA repetitively despite its effectiveness being blocked by butyrate, and the institution of a severe acidosis and attending complications, both long and short term. We have prospective solutions to many of these issues, and a commitment to see this therapy through to a preclinical demonstration of the long-term tolerability and especially the antitumor activity of the regimen.

1998 DoD IDEA Progress report references

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